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TITLE: Engineered Autologous Stromal Cells for the Delivery of Kringle 5, a Potent Endothelial Cell Specific Inhibitor, for Anti-Angiogenic Breast Cancer Therapy

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13. ABSTRACT (Maximum 200 Words)

Glioblastoma multiforme is one of the most highly vascularized tumors in humans. Therefore, the development of a potent antiangiogenic gene therapy startegy for brain cancer represents an attractive alternative to existing therapeutic interventions and circumvents the existing pitfalls associated with direct recombinant antiangiogenic protein delivery. Several potent inhibitors of the angiogenic process are known. Amongst them, angiostatin, is a cleavage product of human plasminogen encompassing the first four N-terminal kringle structures. Of particular interest is the fifth kringle (K5) of plasminogen that has been found to enhance the angiostatic potency of angiostatin. We propose that the K5 domain may serve as a potent angiostatic agent on its own and that it may act as a useful therapeutic transgene within a cancer gene therapy strategy. To test this hypothesis, we have developed a K5-expressing retroviral vector and have characterized the angiostatic activity of the de novo produced K5 peptide *in vitro* and tested its efficacy *in vivo* using an orthotopic brain cancer model. Upon intracerebral implantation of 10^5 U87-GFP (n=5) and U87-K5-GFP (n=5) glioma cells in nude mice, hematoxylin and eosin-stained brain tissue sections reveal that U87-K5-GFP-implanted mice possess significantly reduced tumor volumes as compared to the mock implanted mice 32 days post-implantation. This evidence suggests that soluble K5 peptide holds promise as an attractive anti-angiogenic therapeutic agent within a cancer gene therapy setting.

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REVISED ANNUAL REPORT

NOTE: The previously submitted statement of work proposed the use of genetically-engineered marrow stromal cells (MSCs) as a cellular vehicle for th edelivery of a potent anti-angiogenic transgene, the human plasminogen kringle 5 domain. Data generated by the principal investigator of this grant ("Statement of Work: Task #1a-d Months 1-18") demonstrates that marrow stromal cells retrovirally gene-modified with soluble kringle 5 do not proliferate as normally expected and appeared unhealthy. In addition, a modified-Boyden chamber migration assay was performed and the results demonstrate that soluble kringle 5 significantly inhibits the migration of marrow stromal cells (Figure 1).

Our laboratory has phenotypically characterized the murine marrow stromal cells (MSCs) by antibody staining followed by flow cytometry analysis. We have previously reported (1) that our *in vitro* culture-expanded adherent MSCs are uniformly fibroblast-like in appearance and express CD44 and CD34, however do not express CD45, CD31, flk1/kdr (VEGF-R2), flt-4 (VEGF-R3) and Tie2 (angiopoietin receptor). Therefore, our preliminary data seems to suggest that soluble K5 inhibits MSCs, a population of cells distinct from endothelial cells. Since our aim was to genetically engineer cells to secrete biologically relevant levels of soluble K5 and subsequently test the efficacy of K5 by transplanting the gene-modified cells for *in vivo* production of K5 as a means to treat advanced breast cancer, we decided to alter the cellular vehicle and gene-modified the cancer itself.

Due to the unexpected toxicity induced by soluble kringle 5 on marrow stromal cells, the principal investigator decided to test the use of human glioma cells retrovirally gene-modified *ex vivo* with soluble kringle 5 as a proof-of-concept experiment to further characterize the mechanism of action of kringle 5 and to assess the potency of soluble kringle 5 in an orthotopic brain cancer model. The brief abstract below describes the data that has been generated thusfar.

INTRODUCTION: Glioblastoma multiforme is one of the most highly vascularized tumors in humans. Therefore, the development of a potent antiangiogenic gene therapy startegy for brain cancer represents an attractive alternative to existing therapeutic interventions and circumvents the existing pitfalls associated with direct recombinant antiangiogenic protein delivery. Several potent inhibitors of the angiogenic process are known. Amongst them, angiostatin, is a cleavage product of human plasminogen encompassing the first four N-terminal kringle structures. Of particular interest is the fifth kringle (K5) of plasminogen that has been found to enhance the angiostatic potency of angiostatin. We propose that the K5 domain may serve as a potent angiostatic agent on its own and that it may act as a useful therapeutic transgene within a cancer gene therapy strategy. To test this hypothesis, we have developed a K5-expressing retroviral vector and have characterized the angiostatic activity of the de novo produced K5 peptide *in vitro* and tested its efficacy *in vivo* using an orthotopic brain cancer model.

BODY:

In Vitro Characterization: Soluble K5 is dependent upon proper disulfide bond conformation to maintain functionality. The human plasminogen K5 domain cDNA, containing a 20 amino acid IL-2 signal sequence and a His-tag (C-terminus), was stably transfected into 293T cells. Single clones were drug-selected and screened for maximal

K5 secretion using anti-His immunoblot analysis. Affinity chromatography was performed using a Nickel-NTA column on pre-cleared conditioned media from 293T-K5 cells. Anti-His immunoblot following SDS-PAGE separation, demonstrated a major band of 14kDa. N-terminal amino acid sequencing indicated proper cleavage of the IL-2 signal sequence directly upstream of Ser20. Analysis by MALDI-ToF mass spectrometry revealed a major peak at 12108Da consistent with the predicted molecular weight of K5. To fully characterize cystenil bridge structure, an orthogonal digestion was carried out wither with agarose-immobilized trypsin followed by incubation with Asp-N endopeptidase or with trypsin alone under reducing and non-reducing conditions. Peptide mapping of non-reduced single and dual protease K5 digests by MALDI-QToF mass spectrometry confirmed the presence of disulfide bridges between Cys17-Cys96, Cys67-Cys91 and Cys38-Cys79.

Anti-Angiogenic Characterization: To assess the endothelial cell inhibitory properties of retrovector-generated K5 peptide, the 363bp human plasminogen K5 domain cDNA – nt1495-nt1734 NCBI LocusLink mRNA Source NM_000301- (InvivoGen, San Diego, CA) was His-tagged at the C terminus and cloned into a bicitronic retroviral vector comprising the enhanced green fluorescent protein reporter gene. Upon transfection of K5 retrovector plasmid into 293GPG retroviral packaging cells, single clones were drug selected and characterized. Kringle 5 retroparticles were used to transducer U87 human glioma cells, whereby single clones were isolated and characterized. Southern blot analysis on K5 transduced U87 glioma cells using a GFP cDNA-specific probe suggests unrearrangement upon integration into the genome of transduced glioma cells. Anti-His immunoblot analysis on conditioned supernatant collected from the K5 transduced human U87 glioma cells revealed a major 14kDa protein consistent with the predicted MW of soluble K5. Human glioma cells retrovirally-engineered to express hK5His significantly suppressed *in vitro* human umbilical vein endothelial cell migration and *in vivo* blood vessel formation following subcutaneous Matrigel^{TM} plug implantation in NOD-SCID mice.

We observed that hK5His-producing matrix-embedded glioma suppresses the recruitment of tumor-infiltrating $CD45^{+}Mac3^{+}Gr1^{+/-}$ macrophages in NOD-SCID mice as compared to matrix-embedded U87-GFP cells **(n=10 for each experimental cohort)** and demonstrate that hK5His blocks glioma-initiated migration of human macrophages *in vitro*. Furthermore, we show that upon intracerebral implantation of 10^5 U87-GFP (n=5) and U87-K5-GFP (n=5) glioma cells in nude mice, hematoxylin and eosin-stained brain tissue sections reveal that U87-K5-GFP-implanted mice possess significantly reduced tumor volumes as compared to the mock implanted mice 32 days post-implantation (Figure 2). Therefore, tumor-targeted K5 expression is capable of effectively suppressing glioma growth and also allows for long-term survival in a majority of U87-K5-GFP-implanted mice **(n=15)** as compared to the control U87-GFP-implanted mice **(n=10)** (Figure 3). **We utilized a GFP-expressing retroviral construct to gene-modify packaging cells which produced GFP-expressing retroviral particles that were then used to gene-modify human U87 glioma cells. GFP-expressing U87 cells served as a control to assess whether the inserted GFP retroviral DNA in it of itself induced any toxicity *in vitro*. We demonstrated *in vitro* (Figure 2) that the GFP-expressing U87**

cells doubled at the same rate as the K5-GFP-expressing U87 cells. Therefore, this eliminates the possibility that infection with the K5-GFP-expressing retroviral DNA caused any nonspecific inhibition. Tumor volume measurements were calculated as follows: After euthanasia, brains were removed and quickly frozen in isopentane chilled with liquid nitrogen. Coronal 10μm sections were prepared and stained with H&E. Digital images were retrieved using an Olympus microscope (Olympus America, Melville, NY). Tumor volumes were calculated using the formula $a \times b^2 \times 0.4$, where *a* represents the longest axis and *b* represents the width perpendicular to this axis.

Stereotactic Intracerebral Surgery: CD1 *nu/nu* female athymic nude mice (6 weeks old; Charles River, Laprairie Co., Quebec, Canada) were anesthetised using a ketamine-xylezine-saline-acepromazine cocktail (100mg/ml, 20mg/ml, 0.9%, 10mg/ml respectively) dosed at 100μl/100g by i.p. injection. Natural Tears (Alcon, Fort Worth, TX) was applied to eyes. Mice were secured in stereotactic apparatus (Kopf Instruments, Tujunga, CA) and incisor bar set to -1.5. Midline incision made on scalp to expose Bregma & Lambda. Coordinates from Bregma were: AP: +0.5, LM: -2.0, DV: -4.4. A burr hole was made and Hamilton syringe (10μl, 26 gauge needle, Hamilton Co., Reno, NV) was gently lowered. The U87-GFP control and U87-K5-GFP test cell suspension (1×10^5 cells in 3μl of HBSS) was manually injected at a rate of 0.25μl per 3 minutes for a total of 36 minutes. The needle remained in place an extra 10 minutes before slowly retracting. Vetbond tissue adhesive (3M, Boston, MA) was used to close edges of incision site.

The orthotopic implantation was performed by Zafiro Koty, an experienced research assistant, in collaboration with Dr. Josephine Nalbantoglu at the Montreal Neurological Institute. Since the animal work for this part of the project was not physically performed at the Lady Davis Institute for Medical Research, the PI did not need to amend the animal protocol.

The sources of all reagents utilized in the study are included in the *Materials & Methods* of the manuscript. Upon manuscript submission, the PI will provide a copy to your office if necessary.

FIGURES:

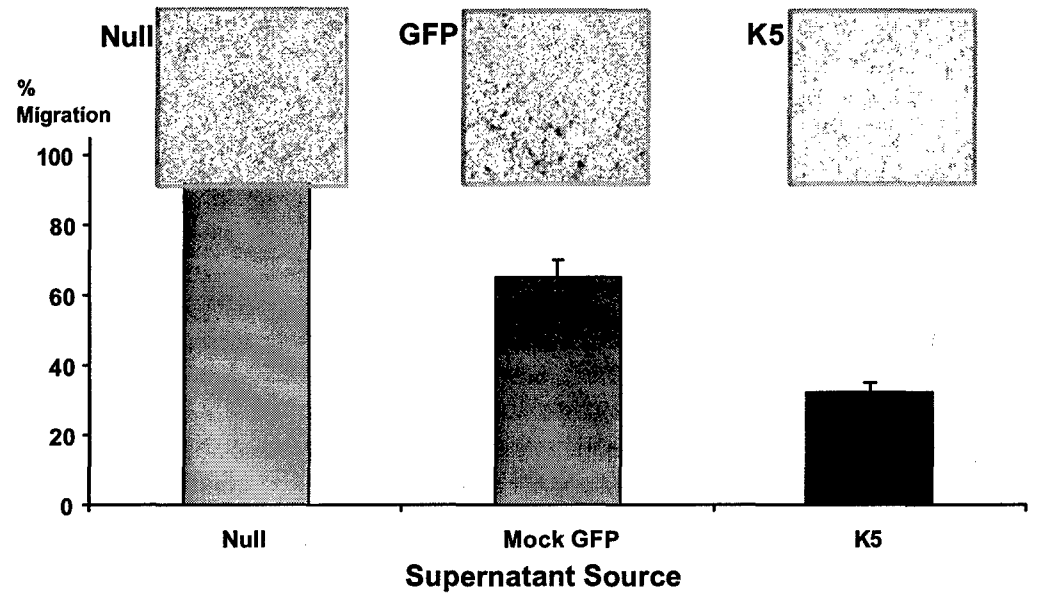

Figure 1: Conditioned media from transduced human glioma cells inhibits MSC migration (4 hours). Soluble K5 inhibits murine MSC migration up to 68% as compared to the conditioned media collected from untransduced U87 cells.

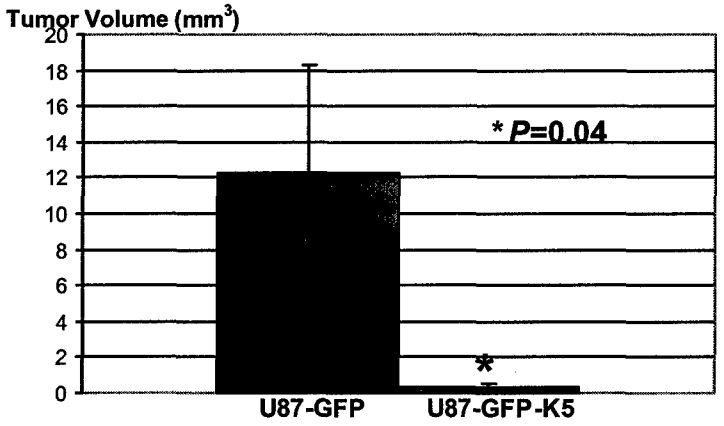

Figure 2: Suppression of intracerebral U87MG glioma growth by soluble K5. Tumor volumes were estimated from post-mortem H&E stained brain sections. The experiment was performed twice with similar results; *bars*, S.E.M.

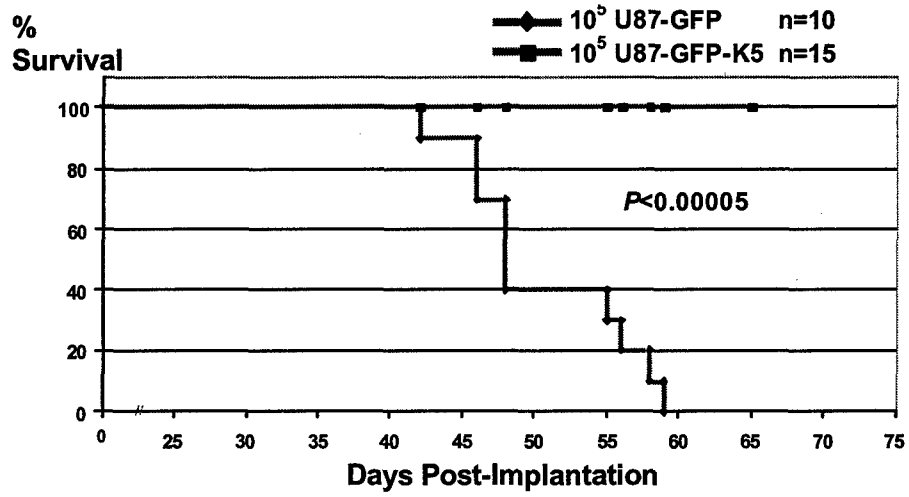

Figure 3: Kaplan-Meier long-term survival curve of mice with implanted with control U87-GFP (n = 10) and U87-K5-GFP (n = 15) cells. By suppressing tumor growth, soluble K5 in turn prolongs the survival of U87-K5-GFP implanted mice.

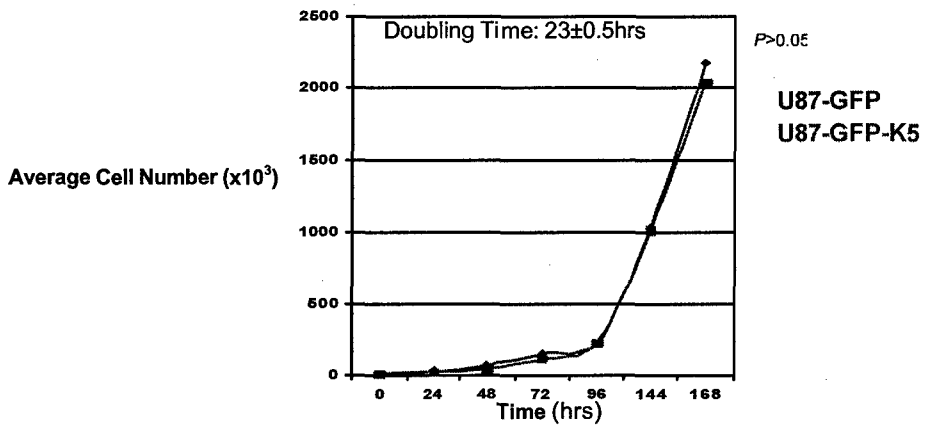

Figure 4: Proliferation rate of GFP-transduced U87 cells is comparable to that of K5-transduced U87 cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Plasminogen K5 domain adopts spontaneously a tertiary structure consistent with native conformation when expressed by genetically-engineered eukaryotic cells.
- Results reveal that K5 gene-modified human glioma cells are capable of appropriately secreting K5, and that the K5 domain on its own can serve as a

potent angiostatic agent in suppressing endothelial cell migration in vitro, possibly by inducing endothelial cell apoptosis.

- Evidence obtained from the Matrigel implant cellular infiltrate analysis also suggests that soluble K5 may possess novel anti-inflammatory properties.
- Our experimental orthotopic brain cancer model also suggests that soluble K5 peptide hold promise as an attractive anti-angiogenic therapeutic agent within a cancer gene therapy setting.

CONCLUSIONS:

Given the success of the proof-of concept experiment, the principal investigator has generated a bicistronic retroviral vector encoding a K5 dimer to compare the potency of monomeric and dimeric K5 forms both in vitro and in vivo. The rationale underlying this approach is such that the dimeric form may form a tertiary structure which would allow for a stronger association with its binding partners. To date, there is no data stipulating the direct mechanism of action for K5. The reagents and data generated in the proof-of principle experiment will be used to guide the principal investigator to plan and carry out the 2nd complementary study. In parallel, using the murine DA3 breast cancer model, the principal investigator will further characterize the mechanism of action of monomeric K5. A revised statement of work for current and future research work was already submitted to your office in December 2004.

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REPORTABLE OUTCOMES

Abstracts:

1. **S. R. Perri,** J. Nalbantoglu, B. Annabi, Z. Koty, L. Lejeune, M. François, M. R. Di Falco, R. Béliveau, and J. Galipeau. Gene-Modified Human Glioma Cells Producing Soluble Human Plasminogen Kringle 5 Peptide Suppress Brain Tumor Progression. Molecular Therapy. May 2004.

This abstract was accepted for poster presentation at the 7th Annual Meeting of the American Society of Gene Therapy (ASGT) held in Minneapolis (June 2004).

2. **S. R. Perri,** J. Nalbantoglu, B. Annabi, Z. Koty, L. Lejeune, M. François, M. R. Di Falco R. Béliveau and J. Galipeau. Gene-Modified Human Glioma Cells Producing Soluble Human Plasminogen Kringle 5 Peptide Suppress Brain Tumor Progression. May 2004.

*This abstract was awarded the **1st Prize ($500) at the 1st Student Poster Competition at the 2nd Montreal Centre for Experimental Therapeutics in Cancer (MCETC) Meeting** held at the Biotechnology Research Institute.*

3. **S. R. Perri,** J. Galipeau, S. James and M. R. Di Falco. MALDI-QToF Mass Spectrometry Analysis of Expressed Soluble Kringle 5 Peptide Confirms Expected Disulfide Bridging Conformation. May 2004.

This abstract was accepted for poster presentation at the 4th International Canadian Proteomics Initiative Conference.

4. **S. R. Perri,** J. Nalbantoglu, B. Annabi, Z. Koty, R. Béliveau and J. Galipeau. Kringle 5, a Novel and Potent Endothelial Cell-Specific Inhibitor for Anti-Angiogenic Gene Therapy of Cancer. November 2003.

*This abstract was **awarded the 1st Prize ($1500) at the 2003 International BioNorth Student Poster Competition** in Ottawa.*

5. **S. R. Perri,** J. Nalbantoglu, B. Annabi, Z. Koty, R. Béliveau and J. Galipeau. Kringle 5, a Novel and Potent Endothelial Cell-Specific Inhibitor for Anti-Angiogenic Gene Therapy of Cancer. October 2003.

*This abstract was **selected among the 12 best submitted abstracts across Canada** to be presented at the 2003 BioContact meeting in Quebec City.*

7. **S. R. Perri,** B. Annabi, R. Béliveau and J. Galipeau. Kringle 5, a Novel and Potent Endothelial Cell-Specific Inhibitor for Anti-Angiogenic Gene Therapy of Cancer. Molecular Therapy. May 2003.

This abstract was accepted for poster presentation at the 6th Annual Meeting of the American Society of Gene Therapy (ASGT) held in Washington D.C. (June 2003).